

# The Role of Diacylglycerol Kinase $\zeta$ and Phosphatidic Acid in the Mechanical Activation of Mammalian Target of Rapamycin (mTOR) Signaling and Skeletal Muscle Hypertrophy

Jae-Sung Yu

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## Introduction

This study aims to find a mechanism for the translation of muscle stimulus to a chemical signal and its affect on muscle size.

## Conclusions

Phospholipase D is not the primary translator of muscle stretch/stimulation into phosphatidic acid. Diacylglycerol Kinase seems to be a greater contributor to translating muscle stretch to phosphatidic acid.

Muscle stretch is translated, mostly, by an mTOR dependent manner.

Increased Diacylglycerol Kinase leads to increases in muscle cell size.

## Amendments

## Study Design & Additional Information

The researchers used mouse muscle cells that were on a plate and ran experiments. They also took out whole muscles from mice and mounted them in a way to induce stretch and made measurements. Then, they used a variety of inhibitors and stimulators, with and without muscle stretch, to measure varying proteins involved in protein synthesis. They also used some mice that were genetically deficient (as in, they couldn't produce their own) diacylglycerol kinase (known as a knockout mouse).

Rapamycin: Inhibits mTOR  
TPA: Stimulates phospholipase D  
FIP1: Inhibits phospholipase D.

### Additional Information:

Phospholipase D produces phosphatidic acid, and Diacylglycerol Kinase does, as well. Diacylglycerol Kinase uses diacylglycerol to produce phosphatidic acid (PLD does not). Phosphatidic acid binds mTOR, which then phosphorylates (activates) the protein called p70, which then (presumably) increases muscle cell size by stimulating protein synthesis.

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## The Role of Diacylglycerol Kinase $\zeta$ and Phosphatidic Acid in the Mechanical Activation of Mammalian Target of Rapamycin (mTOR) Signaling and Skeletal Muscle Hypertrophy\*

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Jae-Sung Yu<sup>1,5</sup>, Hannah C. Lincoln<sup>1</sup>, Chan-Ran Kim<sup>1</sup>, John W. Frey<sup>2</sup>, Craig A. Goodman<sup>3</sup>, Xiao-Ping Zhong<sup>6</sup>, and Troy A. Hornberger<sup>1,5,1</sup>

From the <sup>1</sup>Program in Cellular and Molecular Biology and <sup>2</sup>Department of Comparative Biosciences in the School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706 and the <sup>3</sup>Division of Allergy and Immunology, Department of Pediatrics, and <sup>4</sup>Department of Immunology, Duke University, Medical Center, Durham, North Carolina 27710

**Background:** Diacylglycerol kinases (DGKs) synthesize phosphatidic acid (PA), and PA can activate growth-regulatory mTOR signaling.

**Results:** The  $\zeta$  isoform of DGK is necessary for a mechanically induced increase in PA-mTOR signaling, and overexpression of DGK $\zeta$  induces skeletal muscle hypertrophy.

**Conclusion:** PA synthesized by DGK $\zeta$  regulates the mechanical activation of mTOR signaling and hypertrophy.

**Significance:** DGK $\zeta$  is a potential target for treating muscle atrophy/wasting.

The activation of mTOR signaling is essential for mechanically induced changes in skeletal muscle mass, and previous studies have suggested that mechanical stimuli activate mTOR (mammalian target of rapamycin) signaling through a phospholipase D (PLD)-dependent increase in the concentration of phosphatidic acid (PA). Consistent with this conclusion, we obtained evidence which further suggests that mechanical stimuli utilize PA as a direct upstream activator of mTOR signaling. Unexpectedly though, we found that the activation of PLD is not necessary for the mechanically induced increases in PA or mTOR signaling. Motivated by this observation, we performed experiments that were aimed at identifying the enzyme(s) that promotes the increase in PA. These experiments revealed that mechanical stimulation increases the concentration of diacylglycerol (DAG) and the activity of DAG kinases (DGKs) in membranous structures. Furthermore, using knock-out mice, we determined that the  $\zeta$  isoform of DGK (DGK $\zeta$ ) is necessary for the mechanically induced increase in PA. We also determined that DGK $\zeta$  significantly contributes to the mechanical activation of mTOR signaling, and this is likely driven by an enhanced binding of PA to mTOR. Last, we found that the overexpression of DGK $\zeta$  is sufficient to induce muscle fiber hypertrophy through an mTOR-dependent mechanism, and this event requires DGK $\zeta$  kinase activity (*i.e.* the synthesis of PA). Combined, these results indicate that DGK $\zeta$ , but not PLD, plays an important role in mechanically induced increases in PA and mTOR signaling. Furthermore, this study suggests that DGK $\zeta$  could be a fundamental component of the mechanism(s) through which mechanical stimuli regulate skeletal muscle mass.

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<sup>1</sup> To whom correspondence should be addressed: Dept. of Comparative Biosciences, 2015 Linden Dr., Madison, WI 53706; Tel: 608-890-2174; Fax: 608-890-2174; E-mail: thornb1@svm.vetmed.wisc.edu.

Mechanical stimuli play a critical role in the regulation of skeletal muscle mass, and the maintenance of muscle mass contributes significantly to health and issues associated with quality of life (1, 2). However, the mechanism(s) via which mechanical stimuli are converted into the molecular events that regulate muscle mass remain poorly defined. Nevertheless, advancements in our understanding are being made, and it is becoming increasingly evident that a protein kinase called the mammalian (or mechanistic) target of rapamycin (mTOR)<sup>2</sup> is a key determinant in the mechanical regulation of muscle mass. For example, many studies have demonstrated that mechanical stimulation leads to the activation of mTOR signaling, and its inhibition with the drug rapamycin prevents mechanically induced increases in muscle mass (3–5). Furthermore, it has been shown that the activation of mTOR signaling is sufficient to induce an increase in muscle fiber size (*i.e.* hypertrophy) (6). These observations are important because they provide the cornerstone (*i.e.* mTOR signaling) for investigations that are aimed at developing pharmacological treatments that can mimic the effects of mechanical stimuli on muscle mass. However, to develop such treatments, we will first need to understand how mechanical stimuli regulate mTOR signaling.

Based on previous studies, it has been concluded that mechanical stimuli activate mTOR signaling through a unique

<sup>2</sup> The abbreviations used are: mTOR, mammalian (or mechanistic) target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; CSA, cross-sectional area; DAG, diacylglycerol; DGK, diacylglycerol kinase; EDL, extensor digitorum longus; FIP1, 5-fluoro-2-indolyl des-chloroallopemide; FRB, FKBP12-rapamycin binding; LEL, late endosomal/lysosomal structures; OG, octyl glucoside; p70<sup>S6</sup>, ribosomal S6 kinase; PA, phosphatidic acid; CB PA, 1,2-dioctanoyl-sn-glycero-3-phosphate; PC, phosphatidylcholine; PLD, phospholipase D; PS, phosphatidylserine; PtdBut, phosphatidylbutanol; Raptor, regulatory associated protein of mTOR; Rheb, Ras homologue enriched in brain; TA, tibialis anterior; TPA, 12-O-tetradecanoylphorbol-13-acetate; Lo, optimal length.

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mechanism that does not require typical candidates such as PI3K, protein kinase B, ERK, exogenous nutrients, or intracellular calcium (7–11). However, the identity of the unique mechanism remains unknown (4). Nonetheless, an emerging body of evidence suggests that phosphatidic acid (PA) may play a key role in this process. For instance, various forms of mechanical stimuli can promote an increase in the intracellular concentration of PA (8, 10, 12). Furthermore, just like mechanical stimulation, PA can activate mTOR signaling via a PI3K- and ERK-independent mechanism (8, 11). It has also been shown that PA can bind to the FKBP12-rapamycin binding (FRB) domain of mTOR, and it can directly activate mTOR kinase activity *in vitro* (11, 13, 14). Based on these points, it has been proposed that a mechanically induced increase in PA could lead to an enhanced binding of PA to mTOR and in turn facilitate the activation of mTOR signaling and ultimately muscle growth.

The potential role of PA in the mechanical activation of mTOR signaling was first exposed in 2006 when it was shown that: (i) mechanical stretch induces a transient increase in the activity of phospholipase D (PLD), (ii) the use of 1-butanol to inhibit the synthesis of PA by PLD completely prevents the mechanically induced increase in PA and mTOR signaling, and (iii) mTOR signaling in mechanically stimulated muscles exhibits resistance to the inhibitory effects of rapamycin (10). With regard to this last point, it is important to consider that rapamycin forms a complex with the FKBP12 protein, and like PA, this complex can bind to the FRB domain in mTOR (an event that is responsible for the inhibitory effects of rapamycin on mTOR signaling) (15, 16). Furthermore, it has been suggested that PA and the rapamycin-FKBP12 complex can compete with each other for binding to the FRB domain, and due to this competition, an enhanced binding of PA to the FRB domain would be expected to induce resistance to the inhibitory effects of rapamycin (17, 18). For these reasons it has been concluded that the rapamycin resistance that is observed in mechanically stimulated muscles indicates that mechanical stimulation promotes an enhanced binding of PA to mTOR.

A potential role of PA in the mechanical activation of mTOR signaling was further explored in a subsequent study that employed a bout of eccentric contractions as the source of mechanical stimulation (8). Again, it was determined that 1-butanol prevents the mechanical activation of mTOR signaling. Thus, the results from this study provided further support for the conclusion that a PLD-dependent increase in PA is necessary for the mechanical activation of mTOR signaling. However, more recent studies have shown that many of the biological effects of 1-butanol cannot be attributed to the inhibition of the synthesis of PA by PLD (19–21). Moreover, the time course of the mechanically induced increase in PLD activity does not adequately correspond with the increase in PA, and hence, it has been argued that a mechanically induced increase in PLD activity may not fully explain how mechanical stimuli promote an increase in PA (4). In other words the potential role of PLD and/or PA in the mechanical activation of mTOR signaling remains a subject of debate. Therefore, the initial aim of the current study was to re-evaluate the role that PLD plays in controlling the mechanically induced changes in PA and mTOR signaling. Surprisingly, our results demonstrated that changes

in PLD activity are not required for these events. Instead, we obtained evidence which indicates that the  $\zeta$  isoform of diacylglycerol kinase (DGK $\zeta$ ), an enzyme that synthesizes PA via the phosphorylation of diacylglycerol (DAG), is largely responsible for the mechanically induced increase in PA and significantly contributes to the mechanical activation of mTOR signaling. Furthermore, we determined that the overexpression of DGK $\zeta$  is sufficient to induce hypertrophy, and this effect occurs through a rapamycin-sensitive mechanism that requires DGK $\zeta$  kinase activity (*i.e.* the synthesis of PA). Combined, these results highlight a novel role for DGK $\zeta$  but not PLD, in the mechanical activation of PA-mTOR signaling and reveal new insights into the potential mechanism(s) through which mechanical stimuli ultimately regulates muscle mass.

### EXPERIMENTAL PROCEDURES

**Materials**—PA (1,2-dioctanoyl-*sn*-glycero-3-phosphate (C8 PA)), phosphatidylcholine (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC)), DAG (1-2-dioleoyl-*sn*-glycerol), and phosphatidylbutanol (1,2-dioleoyl-*sn*-glycero-3-phosphobutanol (PtdBut)) were purchased from Avanti Lipids (Alabaster, AL). Phosphatidylserine (1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (PS)) were purchased from Echelon Biosciences (Salt Lake City, UT). [<sup>3</sup>H]Myristic acid and [<sup>32</sup>P]ATP were purchased from Perkin Elmer (Waltham, MA). Rapamycin was purchased from LC laboratories (Woburn, MA). 5-Fluoro-2-indolyl des-chlorohalopemide (FIP) was purchased from Sigma. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), and R59949 was purchased from Enzo Life Sciences (Farmingdale, NY). These drugs were all dissolved in DMSO to make stock solutions.

Rabbit anti-ribosomal S6 kinase (p70<sup>S6k</sup>), anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-ERK1/2, anti-regulatory-associated protein of mTOR (Raptor), anti-mTOR, anti-GFP, and anti-lactate dehydrogenase (LDH) antibodies were purchased from Cell Signaling (Danvers, MA). Rabbit anti-phospho-p70<sup>S6k</sup> (Thr-389), goat anti-DGK $\zeta$ , and FITC-conjugated goat anti-rat IgG antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Rat anti-HA antibody was purchased from Roche Applied Science. Rabbit anti-laminin antibody was purchased from Sigma. HRP-conjugated anti-rabbit IgG antibody was purchased from Vector Laboratories (Burlingame, CA). HRP-conjugated anti-mouse IgG, HRP-conjugated anti-rat IgG, and DyLight 594-conjugated anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

**Plasmid Constructs and Purification**—pEGFP-C3 (GFP) was purchased from Clontech (Mountain View, CA). pRK5-myc-p70<sup>S6k</sup>-glutathione transferase (GST-p70<sup>S6k</sup>) was kindly provided by Dr. Karyn Esser (University of Kentucky, Lexington, KY). YFP-tagged Ras homologue enriched in brain (YFP-Rheb) was obtained from Dr. Kun-Liang Guan (University of California San Diego, La Jolla, CA). pHA3-DGK $\zeta$  with three tandem N-terminal HA epitope tags (HA-WT-DGK $\zeta$ ) was a generous gift from Dr. Matthew Topham (University of Utah, Salt Lake City, UT). pHA3-kinase dead DGK $\zeta$  (HA-KD-DGK $\zeta$ ) was gen-

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erated from the pHA3-DGK $\zeta$  via a guanine-to-adenosine change (22) at nucleotide 1756 of DGK $\zeta$  (NCBI Reference Sequence NM\_001105540.1) using a QuikChange Lightning Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). All plasmid DNA was grown in DH5 $\alpha$  *Escherichia coli*, purified with an EndoFree plasmid kit (Qiagen, Valencia, CA), and resuspended in sterile PBS.

**Animals**—Wild-type FVB/N and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA), Taconic (Hudson, NY), or The Jackson Laboratory (Bar Harbor, MA). DGK $\zeta^{-/-}$  C57BL/6 mice have been described previously (23) and were kindly provided by Dr. Xiao-Ping Zhong (Duke University, Durham, NC). All animals were housed in a room maintained at 25 °C with a 12-h:12-h light:dark cycle and received food and water *ad libitum*. 8–10-Week-old animals were used for all experiments, and they were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) before all surgical procedures. After muscle collection, mice were sacrificed by cervical dislocation under anesthesia. For all non-histochemical analyses, the muscles were immediately frozen in liquid nitrogen at the end of the experimental procedures. All methods were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison under protocol # V01324.

**Ex Vivo Mechanical Stimulation**—Mouse extensor digitorum longus (EDL) muscles were mechanically stimulated in an *ex vivo* organ culture system that consisted of a refined myograph apparatus (Kent Scientific, Torrington, CT) and an organ culture bath as previously described (7). Briefly, the proximal and distal tendons of EDL muscles were connected to a micro-manipulator and the lever arm of a force transducer, respectively. Then the length of the muscle was adjusted until a passive tension of 13.5 mN was obtained (note: preliminary studies demonstrated that the optimal length (Lo) of EDL muscles was obtained at 13.5 mN). The muscles were then subjected to intermittent 15% passive stretch as a source of mechanical stimulation or held static at Lo as a control condition. The bath incubation media consisted of Krebs-Henseleit buffer (120 mM NaCl, 4.8 mM KCl, 25 mM NaHCO $_3$ , 2.5 mM CaCl $_2$ , 1.2 mM KH $_2$ PO $_4$ , 2 mM MgSO $_4$ , and 5 mM HEPES) supplemented with 1 $\times$  minimum essential medium amino acid mixture (Invitrogen) and 25 mM glucose. The medium was maintained at 37 °C with continuous 95% O $_2$  and 5% CO $_2$  gassing, and it was exchanged with fresh medium at 30 min intervals.

**Skeletal Muscle Transfection and Rapamycin Injections**—Mouse tibialis anterior (TA) muscles were transfected by electroporation as previously described (6). In brief, a small incision was made through the skin covering the TA muscle. A total of 30–50  $\mu$ g of plasmid DNA (GFP, HA-WT-DGK $\zeta$ , or HA-KD-DGK $\zeta$ ) solution was then injected into the proximal (6  $\mu$ l) and distal (6  $\mu$ l) ends of the muscle belly with a 27-gauge needle. After the injections, 2 stainless steel pin electrodes (1-cm gap) connected to an ECM 830 electroporation unit (BTX/Harvard Apparatus, Holliston, MA) were laid on top of the proximal and distal myotendinous junctions. Then, eight 20-ms square-wave electric pulses at a frequency of 1 Hz were delivered onto the muscle with a field strength of 160 V/cm. After the electroporation procedure, incisions were closed with Vetbond surgical

glue, and the animals were allowed to recover for 3 or 7 days. In some experiments vehicle or rapamycin solution was administered into the animals via intraperitoneal injections immediately after the electroporation procedure. Rapamycin solution was prepared by dissolving the appropriate volume of the stock solution (in DMSO) that was needed to inject mice with 1.5 or 4.5 mg/kg of body weight in 200  $\mu$ l of PBS. For the vehicle control solution, an equivalent amount of DMSO was dissolved in 200  $\mu$ l of PBS. These injections were repeated every 24 h.

**Cell Culture and Transfection**—C2C12 myoblasts were cultured in growth media consisting of high glucose DMEM (HyClone, Logan, UT) supplemented with antibiotics and antimycotics (100  $\mu$ g/ml streptomycin, 100 units/ml penicillin, and 0.25  $\mu$ g/ml amphotericin) and 10% fetal bovine serum (Invitrogen). For mTOR kinase activity assay, C2C12 myoblasts stably expressing FLAG-tagged mTOR (FLAG-mTOR) were obtained from Dr. Jie Chen (University of Illinois, Urbana, IL) and cultured in the growth medium that was further supplemented with 0.2 mg/ml G418 (HyClone). For stimulation experiments, the growing myoblasts were plated on 6-well dishes at 40–60% confluence. During this time, if needed, cells were co-transfected with 2  $\mu$ g of GFP, HA-WT-DGK $\zeta$ , or HA-KD-DGK $\zeta$  and 0.2  $\mu$ g of GST-p70 $^{S6}$  constructs by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The following day the cells were serum-starved in the absence of any antibiotics and antimycotics before being subjected to experimental treatments. Cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO $_2$ .

**Fractionation**—Frozen muscles were homogenized with a Polytron homogenizer in ice-cold fractionation buffer (20 mM Tris (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 1 mM Na $_2$ VO $_4$ , 1 mM PMSE, and 20  $\mu$ g/ml each leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor), and the homogenates were precleared by centrifugation at 1000  $\times$  g (4 °C) for 10 min. The precleared supernatants (whole lysate) were further centrifuged at 100,000  $\times$  g (4 °C) for 1 h to separate the soluble supernatants (cytosolic fraction) and insoluble pellets (crude membrane fraction) as previously described (24). The membrane pellets were washed once and resuspended in the ice-cold fractionation buffer for DGK activity assay or Laemmli buffer for Western blot analysis. The protein concentration in each whole lysate sample was determined with the DC protein assay kit (Bio-Rad) and used to control the loading amounts of cytosolic and/or membrane protein in the DGK activity assay and Western blot analysis.

**In Vitro DGK Activity Assay**—DGK activity was measured by the standard octyl glucoside (OG)/PS-mixed micelle assay as previously described (25). In brief, OG/PS-mixed micelles were prepared by resuspending dried DAG and PS in 5 $\times$  OG buffer (275 mM OG, 1 mM diethylenetriaminepentaacetic acid) with vortexing and sonication. Then the reaction was initiated by adding 10  $\mu$ l of each diluted sample into 90  $\mu$ l of reaction mixture (20  $\mu$ l of mixed micelles added to 70  $\mu$ l of reaction buffer), which yielded a final concentration of (55 mM OG, 0.25 mM DAG (0.87 mol% in micelles), 1 mM PS, 50 mM imidazole (pH 6.6), 50 mM NaCl, 12.5 mM MgCl $_2$ , 1 mM EGTA, 0.2 mM diethylenetriaminepentaacetic acid, 1 mM DTT, 500  $\mu$ M ATP, and 1

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$\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP). After a 30-min incubation at 25 °C, the reaction was terminated by the addition of 0.45 ml chloroform-methanol 1:2 (v/v) and 0.15 ml of 1% perchloric acid. After vortexing, 0.15 ml of chloroform and 0.15 ml of 1% perchloric acid were added to the mixture and vortexed again. The mixture was centrifuged at 2000  $\times$  g for 5 min, and the lower organic phase was washed twice with 1% perchloric acid. [ $\gamma$ - $^{32}\text{P}$ ]PA in the organic phase was separated on LK5D silica gel plates by TLC with a solvent system consisting of ethyl acetate-isooctane-acetic acid-water 13:2:3:10 (v/v) as previously described (10). The amount of [ $\gamma$ - $^{32}\text{P}$ ]PA was then visualized and quantified with a Storm PhosphorImager (GE Healthcare).

**In Vitro mTOR Kinase Activity Assay**—Growing C2C12 myoblasts stably expressing FLAG-mTOR were plated and then maintained in G418-free growth media. Upon confluence, the cells were serum-starved overnight and then collected in either ice-cold CHAPS lysis buffer (40 mM HEPES (pH 7.4), 2 mM EDTA, 0.3% CHAPS, 10 mM sodium pyrophosphate, 10 mM  $\beta$ -glycerophosphate, and 1 tablet of EDTA-free protease inhibitors (Roche Applied Science) per 25 ml) or Triton X-100 lysis buffer (40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM  $\beta$ -glycerol phosphate, 25 mM NaF, 1 mM  $\text{Na}_2\text{VO}_4$ , 10  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM PMSF). Fresh cell lysates were centrifuged at 2500  $\times$  g for 5 min, and 100–150  $\mu\text{g}$  of protein from the supernatant was immunoprecipitated for the FLAG tag by incubating with 10  $\mu\text{l}$  of EZview red ANTI-FLAG M2-agarose affinity gel beads (Sigma) at 4 °C for 2 h. After the incubation, the beads were pelleted by centrifugation at 500  $\times$  g for 30 s and washed with fresh ice-cold wash buffer (40 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.3% CHAPS, 10 mM sodium pyrophosphate, 10 mM  $\beta$ -glycerophosphate, and 1 tablet of EDTA-free protease inhibitors per 25 ml) 3 times and kinase wash buffer (25 mM HEPES (pH 7.4) and 20 mM KCl) 2 times.

For lipid stimulations, C8 PA vesicles (50% C8 PA and 50% PC) or PC vesicles (100% PC) were prepared by drying lipids under nitrogen gas and dissolving in vesicle buffer (150 mM NaCl and 10 mM Tris (pH 8.0)) with 5 min of sonication at a concentration of 6 mM. The lipid vesicles were then diluted to 150  $\mu\text{M}$  in 1.5 $\times$  reaction buffer (see below), and 10  $\mu\text{l}$  of this solution was incubated with the immunoprecipitates at 30 °C for 15 min. The mTOR kinase reaction toward purified GST-p70<sup>S6k</sup> (11) was initiated by adding 5  $\mu\text{l}$  of 750  $\mu\text{M}$  ATP into the immunoprecipitates, which yields a final mTOR kinase assay buffer that contains (25 mM HEPES (pH 7.4), 50 mM KCl, 10 mM  $\text{MgCl}_2$ , 250  $\mu\text{M}$  ATP, 50 ng of GST-p70<sup>S6k</sup>, and 100  $\mu\text{M}$  C8 PA or PC vesicles). After 20 min of incubation, the reaction was terminated by the addition of 25  $\mu\text{l}$  of 2 $\times$  Laemmli buffer. Samples were boiled for 5 min and subjected to Western blot analysis as described below.

**Western Blot Analysis**—Frozen muscles were homogenized in the ice-cold Triton X-100 lysis buffer and the whole homogenates were used for analysis. Myoblasts were lysed in the ice-cold Triton X-100 buffer and centrifuged at 500 g (4 °C) for 5 min, and the supernatant was used for further analyses. The protein concentration in each sample was determined with the DC protein assay kit.

Equal amounts of protein from each sample were dissolved in 2 $\times$  Laemmli buffer before being subjected to SDS-PAGE. After the electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% milk in TBST (TBS with 1% Tween 20) for 1 h, and probed with the indicated primary antibodies overnight at 4 °C. The membranes were then washed for 30 min in TBST and incubated with the appropriate HRP-conjugated secondary antibodies for 45 min at room temperature. After 30 min of washing in TBST, the blots were incubated in regular ECL (Pierce) or ECL Prime (GE Healthcare) for 5 min at room temperature. Images of the blots were either captured on film or with a Chemi410 camera mounted to a UVP Autochemi system (UVP, Upland, CA). Once the appropriate image was captured, the membranes were stained with Coomassie Blue to verify equal loading throughout all lanes. Densitometric measurements of each blot were carried out using ImageJ (National Institutes of Health).

**Immunohistochemical Analysis**—Transfected muscles were subjected to immunohistochemistry as previously described (6). Briefly, collected muscles were incubated in PBS containing 4% paraformaldehyde with gentle rocking at 4 °C for 30 min. The muscles were then submerged in optimal cutting temperature compound (Tissue-Tek; Sakura, Torrance, CA) at resting length and frozen in liquid nitrogen-chilled isopentane. Cross-sections (10  $\mu\text{m}$  in thickness) from the mid-belly of the muscle were obtained with a cryostat and fixed in  $-20$  °C acetone for 10 min. Sections were warmed to room temperature for 5 min and then rehydrated with cool steam followed by a 15-min incubation in PBS. Note that in experiments that did not include GFP-transfected muscles, the above paraformaldehyde and steam vapor treatments were omitted as in Goodman *et al.* (26). The rehydrated sections were then incubated in solution A (PBS containing 0.5% BSA and 0.5% Triton X-100) for 20 min and probed with the indicated primary antibodies dissolved in solution A for 45 min at room temperature. Sections were washed with PBS and then incubated with the appropriate fluorophore-conjugated secondary antibodies dissolved in solution A for 1 h at room temperature. Finally, the sections were washed with PBS, and images of the different fluorophores were captured with a DS-QImc camera on an 80i epifluorescence microscope (both from Nikon, Tokyo, Japan). The monochrome images were merged with NIS-Elements D image analysis software (Nikon), and the average cross-sectional area (CSA) of 35–120 randomly selected fibers per sample was measured by tracing the periphery of individual fibers along the laminin stain. All analyses were performed by investigators that were unaware of the sample identification.

**Analysis of PLD Activity and Lipid Concentrations**—PLD activity and PA, and DAG concentrations in skeletal muscles *ex vivo* were measured with modifications of previously described methods (10). Briefly, EDL muscles were prelabeled in the organ culture system with media containing [ $^3\text{H}$ ]myristic acid (2.5  $\mu\text{Ci}/\text{ml}$ ) for 2 h and then subjected to experimental treatments. For the measurement of PLD activity, the PLD-mediated transphosphatidyl reaction was initiated by incubating the muscles with fresh medium containing 0.5% 1-butanol during the final 15 min of the treatments. The muscles were homogenized with a Polytron in chloroform-methanol 2:1

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(v/v), and total lipids were extracted according to Folch *et al.* (56). The extracted lipids were combined with 10  $\mu$ g of PtdBut, PA, or DAG standards and then subjected to TLC as described in the DAG activity assay section. The solvent system consisted of ethyl acetate:isooctane:acetic acid:water 13:2:3:10 (v/v) for separation of PtdBut and PA or hexane:diethyl ether:acetic acid 50:50:1 (v/v) for separation of DAG. The standard PtdBut, PA, or DAG spots containing the  $^3$ H-labeled PtdBut, PA, or DAG, respectively, was visualized by iodine staining and scraped off the TLC plate to count the amount of radioactivity by liquid scintillation spectrometry. An aliquot of the extracted lipids was also subjected to the liquid scintillation spectrometry to count radioactivity in the total lipids. Final calculations for PLD activity, PA, and DAG were made by dividing the amount of radioactivity in the Ptdbut, PA, or DAG spot by the amount of radioactivity in the total lipid extract.

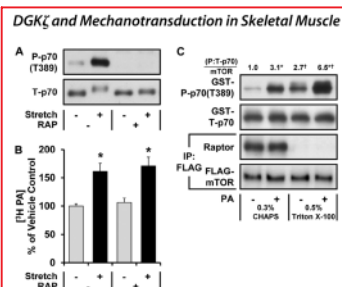
For analysis of PLD activity and PA in cells, myoblasts were prelabeled in serum-free medium containing [ $^3$ H]myristic acid (2.5  $\mu$ Ci/ml) overnight and then washed twice with fresh serum-free medium. After an additional 2-h incubation in serum-free medium, the cells were subjected to experimental treatments in the presence (for PLD activity) or absence (for PA) of 0.3% 1-butanol during the final 15 min of the treatments. The cells were collected in chloroform-methanol 2:1 (v/v) and then processed as described above.

**Statistical Analysis**—All values are expressed as the means ( $\pm$ S.E. in graphs). Statistical significance was determined by using the student's *t* test (2-tailed, unpaired) or analysis of variance (one-way or two-way) followed by Student-Newman-Keuls post hoc analysis. Differences between groups were considered significant when  $p \leq 0.05$ . All statistical analyses were performed on SigmaStat software (San Jose, CA).

## RESULTS

### Evidence That PA Can Function as a Direct Upstream Activator of mTOR Signaling in Response to Mechanical Stimulation

In this study we employed the same *ex vivo* model of mechanical stimulation that was previously used to expose the potential role of PLD and PA in the mechanical activation of mTOR signaling (10). With this model, it has been shown that mechanical stimulation induces an increase in PA and rapamycin-sensitive mTOR signaling (10, 11); however, whether the increase in PA is driven through a rapamycin-sensitive mechanism has not been addressed. This is an important question because recent studies have revealed that rapamycin-sensitive mTOR can phosphorylate and inhibit the activity of the phosphatidic acid phosphatase called Lipin 1 (27, 28). Thus, it remained possible that the mechanically induced increase in PA could be driven through a pathway that lies downstream, rather than upstream, to the activation of rapamycin-sensitive mTOR. Therefore, to test this we subjected muscles to mechanical stimulation in the presence or absence of rapamycin and then examined the muscles for changes in mTOR signaling and PA. As expected, rapamycin eliminated the mechanical activation of rapamycin-sensitive mTOR signaling as revealed by changes in the phosphorylation of p70<sup>S6k</sup> on the Thr-389 residue (29) (Fig. 1A). However, rapamycin did not affect the mechanically induced increase in PA (Fig. 1B). Based on these



**FIGURE 1. Evidence that PA can function as a direct upstream activator of mTOR signaling in response to mechanical stimulation.** A and B, mouse EDL muscles were held at Lo in an *ex vivo* organ culture system and treated as follows: A, preincubated with 150 nM rapamycin (RAP+) or the vehicle (RAP−, DMSO) for 30 min and then subjected to 90 min of a stretch (Stretch+) or control condition (Stretch−) followed by Western blot analysis for phosphorylated (P) and total (T) p70. B, prelabeled with [ $^3$ H]myristic acid for 2 h. During the final 30 min of the prelabeling, the muscles were incubated with rapamycin or the vehicle as in A and then subjected to 90 min of the stretch or control conditions. The concentration of  $^3$ H-labeled PA was measured and expressed as a percentage of values obtained in the vehicle control samples. C, C2C12 myoblasts stably expressing FLAG-mTOR were serum-starved overnight and collected in either CHAPS or Triton X-100 lysis buffer. The lysates were subjected to immunoprecipitation (IP) for the FLAG-epitope, and then the immunoprecipitates were incubated for 15 min with 150  $\mu$ M PA vesicles (PA+) or 150  $\mu$ M PC vesicles as a control condition (PA−). The kinase activity of mTOR was then assayed with GST-p70 as a substrate. The resulting samples were subjected to Western blot analysis for the indicated proteins, and the phosphorylated/total ratios of GST-p70 were divided by the amount of mTOR in each reaction. These values were then expressed as a ratio of the values obtained in the PC-treated samples collected in CHAPS lysis buffer. The values were obtained from four independent experiments. All values are presented as the mean ( $\pm$ S.E. in graphs,  $n = 3$ –11 per group). \* significantly different from the drug (B)- or lysis buffer (C)-matched control group. † significantly different from the stimulation-matched CHAPS group;  $p \leq 0.05$ .

results, it can be concluded that the mechanically induced increase in PA is driven through a pathway that lies upstream and/or parallel to the activation of the rapamycin-sensitive elements of mTOR signaling.

mTOR is typically found in multiprotein complexes such as the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2). mTORC1 is defined by the presence of a protein called Raptor, whereas mTORC2 contains a protein called the rapamycin-insensitive companion of mTOR (Rictor) but not Raptor. Previous studies have suggested that signaling by mTORC1, but not mTORC2, is responsible for controlling the rapamycin-sensitive phosphorylation of substrates such as p70<sup>S6k</sup> (Thr-389) (30). However, more recent studies have shown that mTOR, in the absence of Raptor, can also regulate p70<sup>S6k</sup> (Thr-389) phosphorylation through a rapamycin-sensitive mechanism (16). In other words it appears that there is an uncharacterized rapamycin-sensitive pool of mTOR that does not involve the classic mTORC1. Furthermore, it was recently proposed that mechanical stimuli might specifically regulate this uncharacterized pool of mTOR rather than mTORC1 (11). Based on this possibility, we wanted to determine if PA could activate mTOR signaling in the absence of Raptor. To test this,

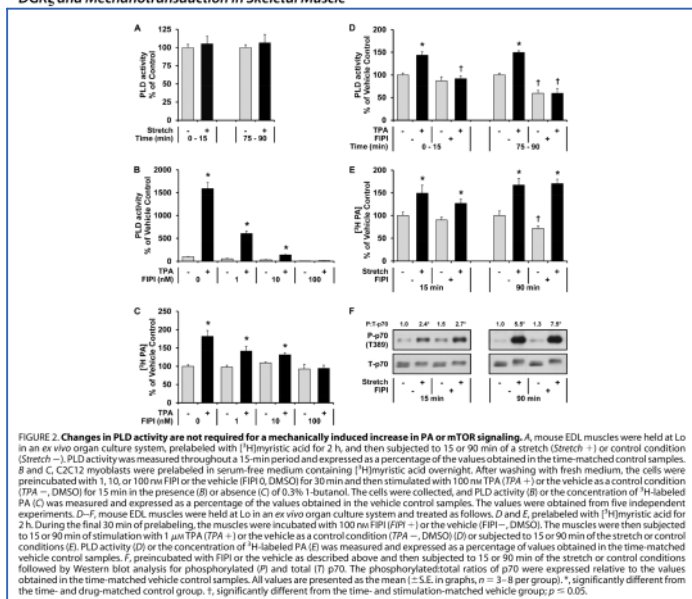
**Figure 1:** [A] The researchers are measuring the amount of phosphorylated p70 (P-p70), which is a downstream marker of mTOR activity. T-p70 is "total" p70. They are using muscle stretch or no stretch, as well as rapamycin (an mTOR inhibitor) or not to measure mTOR activity by how much of total p70 becomes phosphorylated (more phosphorylation indicating more mTOR activity). [B] The researchers are measuring phosphatidic acid concentrations with stretch of the muscle with and without rapamycin (mTOR inhibitor).

### Primary Results:

- Stretch on muscle induces increases in phospho-p70 expression, but not in the presence of Rapamycin.
- Stretch on muscle induces increases in phosphatidic acid, which is preserved in the presence of rapamycin.

**Take Away:** Muscle stimulation/stretch leads to increases in phosphorylation of p70 in an mTOR dependent manner, and phosphatidic acid is also upregulated with muscle stretch, but unaffected by mTOR inhibition, indicating its stimulation is upstream of mTOR.

## DGK $\zeta$ and Mechanotransduction in Skeletal Muscle



**FIGURE 2. Changes in PLD activity are not required for a mechanically induced increase in PA or mTOR signaling.** A, mouse EDL muscles were held at Lo in an ex vivo organ culture system, prelabeled with [ $^3$ H]myristic acid for 2 h, and then subjected to 15 or 90 min of a stretch (Stretch +) or control condition (Stretch -). PLD activity was measured throughout a 15-min period and expressed as a percentage of the values obtained in the time-matched control samples. B and C, C2C12 myoblasts were prelabeled in serum-free medium containing [ $^3$ H]myristic acid overnight. After washing with fresh medium, the cells were preincubated with 1, 10, or 100 nM FIPI or the vehicle (FIPI 0, DMSO) for 30 min and then stimulated with 100 nM TPA (TPA +) or the vehicle as a control condition (TPA -, DMSO) for 15 min in the presence (B) or absence (C) of 0.3% 1-butanol. The cells were collected, and PLD activity (B) or the concentration of  $^3$ H-labeled PA (C) was measured and expressed as a percentage of the values obtained in the vehicle control samples. The values were obtained from five independent experiments. D-F, mouse EDL muscles were held at Lo in an ex vivo organ culture system and treated as follows. D and E, prelabeled with [ $^3$ H]myristic acid for 2 h. During the final 30 min of prelabeling, the muscles were incubated with 100 nM FIPI (FIPI +) or the vehicle (FIPI -, DMSO). The muscles were then subjected to 15 or 90 min of stimulation with 1  $\mu$ M TPA (TPA +) or the vehicle as a control condition (TPA -, DMSO) (D) or subjected to 15 or 90 min of the stretch or control conditions (E). PLD activity (D) or the concentration of  $^3$ H-labeled PA (E) was measured and expressed as a percentage of values obtained in the time-matched vehicle control samples. F, preincubated with FIPI or the vehicle as described above and then subjected to 15 or 90 min of the stretch or control conditions followed by Western blot analysis for phosphorylated (P) and total (T) p70. The phosphorylation/total ratios of p70 were expressed relative to the values obtained in the time-matched vehicle control samples. All values are presented as the mean  $\pm$  S.E. in graphs,  $n = 3-8$  per group. \*, significantly different from the time- and drug-matched control group. †, significantly different from the time- and stimulation-matched vehicle group;  $p = 0.05$ .

we collected C2C12 myoblasts that stably express FLAG-mTOR in CHAPS lysis buffer to preserve the mTOR/Raptor interaction or Triton X-100 lysis buffer to dissociate the mTOR/Raptor interaction. mTOR from the different lysis conditions was then immunoprecipitated and incubated with lipid vesicles composed of 50% C8 PA and 50% PC or 100% PC as a control condition. The immunoprecipitates were then subjected to an *in vitro* mTOR kinase activity assay with p70<sup>S6</sup> as a substrate. In this experiment we confirmed an enhanced mTOR kinase activity in the absence of Raptor as previously demonstrated (Fig. 1C) (31). Importantly, the results also demonstrated that PA can induce an increase in mTOR kinase activity and that Raptor is not required for this effect (Fig. 1C). Combined, the results presented in Fig. 1 establish that a mechanically induced increase in PA could function as a direct upstream activator of mTOR signaling.

**Changes in PLD Activity Are Not Required for a Mechanically Induced Increase in PA or mTOR Signaling**—To reexamine the role of PLD in the mechanical regulation of PA and mTOR

signaling, we first performed measurements of PLD activity in muscles that were prelabeled with [ $^3$ H]myristic acid, which preferentially labels the PLD substrate PC (32). In contrast to the previous study that used [ $^3$ H]arachidonic acid for labeling (10), our results indicated that mechanical stimulation does not alter the activity of PLD toward [ $^3$ H]myristic acid-labeled substrates (Fig. 2A). However, mechanical stimulation was still able to induce an increase in the concentration of [ $^3$ H]myristic acid-labeled PA (Fig. 2E). This observation suggested that changes in PLD activity are not required for a mechanically induced increase in PA.

To further determine if changes in PLD activity are required for a mechanically induced increase in PA, we employed FIPI, which is known to be a more potent and specific inhibitor of PLD than 1-butanol (19). With this compound, we first performed a dose-response analysis in C2C12 myoblasts to identify the minimal concentration of FIPI that was needed to fully inhibit an agonist-induced increase in PLD activity. When TPA was used as a PLD agonist, it was determined that 100 nM FIPI

**Figure 2:** [A] The researchers have taken a muscle out of a mouse and mounted it on a contraction that holds the muscle in place, while in a bath filled with nutrients for the muscle cells, and stretched the muscle for no time (Stretch, -) or 15 minutes or 90 minutes (Stretch, +) to induce muscle contractions. Then, they measured the activity of phospholipase D, which generates phosphatidic acid (see additional notes section for more clarification). [B & C] The researchers are measuring phospholipase D activity with FIPI (a PLD inhibitor) and TPA (a PLD stimulator) in muscle cells (not tissue). [E] Like [A], the researchers have taken muscles out of mice and put them in a nutrient bath while clamped on either end to induce stretch. This time, they also add a PLD stimulator (TPA) or don't, as well as add an inhibitor (FIPI) or don't. [F] The researchers are measuring protein levels of phospho-p70 (the downstream molecule from mTOR) - so, the darker the band/smudge, the more there is (relative to total p70/T-p70). This is all done with stretch (or not) induced on the muscle before measuring, as well as with the PLD inhibitor (FIPI).

### Primary Results

- Stretch does not increase PLD activity.
- FIPI inhibits PLD from increasing activity, but does not suppress basal activity.
- Stretch increases phosphatidic acid production, which is unaffected by PLD inhibition.
- Stretch also increases phospho-p70 production, which is unaffected by PLD inhibition.

**Take Away:** Phospholipase D is not a major player in the increased production of phosphatidic acid when the muscle is stimulated.

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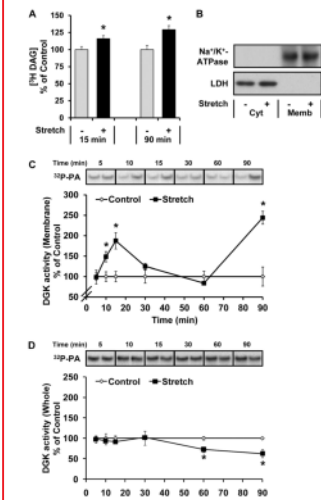
### DGK $\zeta$ and Mechanotransduction in Skeletal Muscle

could completely prevent the TPA-induced increase in both PLD activity and PA (Fig. 2, B and C). We also determined that 100 nM FPLI could completely block the TPA-induced increase in PLD activity when muscles were incubated in the *ex vivo* organ culture system (Fig. 2D).

Having established that 100 nM FPLI could block agonist-induced changes in PLD activity, we next set out to determine if FPLI would impair the ability of mechanical stimuli to induce an increase in PA and mTOR signaling. As shown in Fig. 2E, the increase in PA induced by both 15 and 90 min of mechanical stimulation was not impaired when muscles were treated with FPLI. Furthermore, FPLI did not alter the ability of mechanical stimuli to induce an increase in mTOR signaling (Fig. 2F). When combined, these results demonstrate that mechanical stimuli induce an increase in PA and mTOR signaling via a PLD-independent mechanism.

**Mechanical Stimulation Increases DAG and Membrane DGK Activity**—Our finding that changes in PLD activity are not required for a mechanically induced increase in PA inspired us to search for another enzyme(s) that could contribute to this event. During this search, we realized that several forms of mechanical stimuli, including contractions and stretch, have been reported to induce a rapid increase in the intracellular concentration of DAG (12, 33). Based on these reports, we envisioned the potential for a mechanism in which mechanical stimulation induces an accumulation of DAG, and this in turn would provide an enhanced level of substrate for the DGKs. Because the DGKs can synthesize PA via the phosphorylation of DAG, the net result of the DAG accumulation would be an enhanced synthesis of PA. To begin testing this possibility, we first wanted to determine if our model of mechanical stimulation also promotes an increase in DAG. As shown in Fig. 3A, DAG, just like PA, was significantly elevated after both 15 and 90 min of mechanical stimulation. Next, we asked if the elevated levels of DAG are associated with an increase in DGK activity. Interestingly, we found that mechanical stimulation induced a biphasic increase in membranous DGK activity (Fig. 3, B and C). However, the activity of DGK in whole muscle lysates was not increased by mechanical stimulation (Fig. 3D). Thus, it appears that mechanical stimulation causes a pool of DGK to translocate to membranous structures and/or allosterically activates membrane-associated DGK.

**R59949 Does Not Inhibit the Mechanically Induced Increase in PA or the Activation of mTOR Signaling**—The aforementioned findings prompted us to further explore the potential role of the DGKs in the mechanical regulation of PA and mTOR signaling. To begin this analysis, we first took advantage of the DGK inhibitor R59949. Before describing our results, it is important to mention that the DGK family of enzymes is composed of at least 10 different isoforms, and currently available inhibitors of DGK (R59949 and its weaker analog R59902) preferentially inhibit only the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\theta$  isoforms of DGK (34, 35). Consistent with these points, we found that R59949 (100  $\mu$ M) induces a partial, but significant, inhibition of basal DGK activity (Fig. 4A). However, R59949 did not inhibit the mechanically induced increase in PA or mTOR signaling (Fig. 4, B and C). Based on these results, we have concluded that the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,



**FIGURE 3. Mechanical stimulation increases DAG and membrane DGK activity.** Mouse EDL muscles were held at Lo in an *ex vivo* organ culture system and treated as follows. **A**, prelabeled with [ $^3$ H]myristic acid for 2 h and then subjected to 15 or 90 min of a stretch (Stretch +) or control condition (Stretch -). The concentration of  $^3$ H-labeled DAG was measured and expressed as a percentage of values obtained in the control samples. **B**, subjected to 15 min of the stretch or control conditions and then separated into cytosolic (Cyt) and membrane (Mem) fractions. The different fractions were then subjected to Western blot analysis for Na $^+$ /K $^+$ -ATPase and LDH. **C** and **D**, subjected to 5–90 min of the stretch or control conditions, and then DGK activity ( $^3$ H-PA) in the membrane fraction (**C**) and whole lysates (**D**) was measured and expressed as a percentage of the values obtained in the time-matched control samples. All values are presented as the mean  $\pm$  S.E. ( $n = 3$ –4 per group). \*, Significantly different from the time-matched control group;  $p \leq 0.05$ .

and  $\theta$  isoforms of DGK are not necessary for a mechanically induced increase in PA and mTOR signaling.

**The Mechanically Induced Increase in PA and mTOR Signaling Is Impaired in Muscles from DGK $\zeta$   $^{-/-}$  Mice**—Previous studies have shown that the  $\delta$ ,  $\epsilon$ , and  $\zeta$  isoforms of DGK are highly expressed in skeletal muscle (36). Of these isoforms, we became particularly interested in DGK $\zeta$  because this isoform is resistant to the inhibitory effects of R59949, and in HEK 293 cells it has been reported that the synthesis of PA by DGK $\zeta$  contributes to the serum-induced activation of mTOR signaling (34, 37). Moreover, we were able to further validate this latter point in C2C12 myoblasts by demonstrating that the

**Figure 3:** [A] The researchers are assessing Diacylglycerol (see "additional information section" for more) amount in muscles taken from mice and mounted to induce stretch (or not) for 15 minutes or 90 minutes. [C] The researchers are measuring the activity of the enzyme Diacylglycerol Kinase (DGK), which also produces phosphatidic acid from diacylglycerol (measured in [A]) with and without stretch, but specifically on the membrane of the muscle cells. [D] This is the same experiment as [C], but looking at total DGK, not just membrane bound DGK activity.

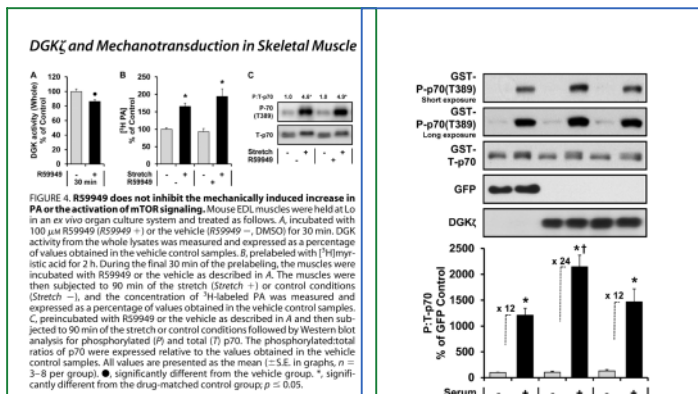
#### Primary Results:

- Diacylglycerol is increased with muscular stretch/stimulation.
- Diacylglycerol Kinase produces more phosphatidic acid when stretch is applied to the muscle, but only membrane bound DGK does this - not found for total.

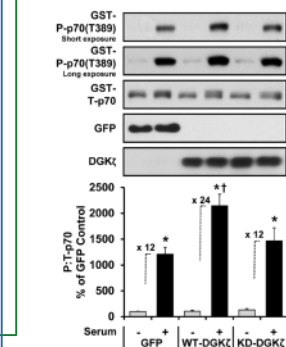
#### Take Away:

Muscle stimulation increases diacylglycerol levels within the muscle, and phosphatidic acid production is increased by diacylglycerol kinase enzymes that are specifically found on the membrane of the muscle cell. This implies DGK produces phosphatidic acid at the membrane of the muscle cell.

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**FIGURE 4. R59949 does not inhibit the mechanically induced increase in PA or the activation of mTOR signaling.** Mouse EDL muscles were held at Lo in an *ex vivo* organ culture system and treated as follows. **A**, incubated with 100  $\mu$ M R59949 (R59949+) or the vehicle (R59949-; DAGK $\zeta$ ) for 30 min. DAG activity from the whole lysates was measured and expressed as a percentage of values obtained in the vehicle control samples. **B**, prelabeled with [<sup>3</sup>H]myristic acid for 2 h. During the final 30 min of the prelabeling, the muscles were incubated with R59949 or the vehicle as described in **A**. The muscles were then subjected to 90 min of the stretch (Stretch+) or control conditions (Stretch-), and the concentration of <sup>3</sup>H-labeled PA was measured and expressed as a percentage of values obtained in the vehicle control samples. **C**, preincubated with R59949 or the vehicle as described in **A** and then subjected to 90 min of the stretch or control conditions followed by Western blot analysis for phosphorylated (P) and total (T) p70. The phosphorylated/total ratios of p70 were expressed relative to the values obtained in the vehicle control samples. All values are presented as the mean  $\pm$  S.E. in graphs,  $n = 3-8$  per group. \* significantly different from the vehicle group; +, significantly different from the drug-matched control group;  $p \leq 0.05$ .



**FIGURE 5. Overexpression of DGK $\zeta$  enhances the serum-induced activation of mTOR signaling in a kinase activity-dependent manner.** C2C12 myoblasts were co-transfected with plasmid DNA encoding GFP, wild type DGK $\zeta$  (WT-DGK $\zeta$ ), or kinase dead DGK $\zeta$  (KD-DGK $\zeta$ ) and GST p70. The following day the cells were serum-starved overnight and then stimulated with 20% fetal bovine serum (Serum+) for 30 min. The cells were collected and then subjected to Western blot analysis for the indicated proteins. The phospho (P)/total (T) ratios of GST-p70 were then expressed as a percentage of the values obtained in the GFP non-stimulated (control, Serum-) samples. Values are presented as the mean  $\pm$  S.E. and were obtained from five independent experiments ( $n = 5-9$  per group). \*, significantly different from the plasmid-matched control group; †, significantly different from the stimulation-matched GFP control group;  $p \leq 0.05$ .

**C and D**). Finally, when mechanically stimulated muscles from WT and DGK $\zeta$ <sup>-/-</sup> mice were treated with increasing doses of rapamycin (0–20 nM), it was determined that mTOR signaling in the DGK $\zeta$ <sup>-/-</sup> muscles was significantly more sensitive to the inhibitory effects of rapamycin (Fig. 6E). As mentioned in the introduction, mTOR signaling in mechanically stimulated muscles exhibits resistance to the inhibitory effects of rapamycin, and this is most likely driven by an enhanced binding of PA to the FRB domain in mTOR. Thus, the results from these experiments not only demonstrate that DGK $\zeta$  is largely responsible for the mechanically induced increase in PA but also suggest that the DGK $\zeta$ -dependent increase in PA leads to an enhanced binding of PA to mTOR and, in-turn, the activation of mTOR signaling.

**Overexpression of DGK $\zeta$  Induces Hypertrophy through a Kinase-dependent and Rapamycin-sensitive Mechanism**—Previous studies have shown that signaling through mTOR is necessary for a mechanically induced hypertrophic response and that the activation of mTOR signaling is sufficient to induce hypertrophy (3, 6). Thus, we wanted to know if an enhanced synthesis of PA by DGK $\zeta$  could also promote a hypertrophic response. To address this question, we transfected TA muscles with plasmid DNA encoding WT-DGK $\zeta$ , a kinase dead mutant of DGK $\zeta$  (KD-DGK $\zeta$ ), or GFP as a control condition. As

**Figure 4:** [A] The researchers are assessing Diacylglycerol Kinase (DAGK) activity (see "additional information section" for more details) in muscles taken from mice and mounted while incubated with a DAGK inhibitor (R59949) for 30 minutes. This inhibitor does not affect all DAGK, it spares a particular DAGK isoform (version) which we will call "Special DAGK" or sDAGK, because they called it something confusing and stupid. [B] This is the same experiment as [A], but they are inducing stretch (or not) on the muscle taken from the mouse and mounted for stretching (Muscle contraction) and they are measuring phosphatidic acid production. [C] The researchers are measuring protein levels of phospho-p70 (the downstream molecule from mTOR) - so, the darker the band/smudge, the more there is (relative to total p70/T-p70). This is all done with stretch (or not) induced on the muscle before measuring, with and without the DAGK inhibitor (that spares sDAGK).

### Primary Results

- DAGK activity is somewhat inhibited by the DAGK inhibitor (R59949).
- Phosphatidic acid production increases with muscle stimulation and that effect is still seen with the DAGK inhibitor.
- phospho-70 is still elevated with the introduction of stretch and the inhibitor.

**Take Away:** With inhibition of other DAG Kinases (DAGK), aside from sDAGK, there is still an increase in phosphatidic acid and phospho-p70.

**Figure 5:** The researchers added a gene to muscle cells that contains the genetic material for a green fluorescent protein (GFP) attached to a gene for the specific diacylglycerol kinase of interest (sDAGK from before), known as WT-DGK. However, they also added a mutated diacylglycerol kinase that does not function, known as KD-DGK. Now, the cells will create green fluorescent protein attached to diacylglycerol kinases of one sort or another. Then, the researchers measured the amount of phosphorylated p70 (remember, the downstream molecule that is activated/phosphorylated by mTOR) relative to total p70 (T-p70). Then, they either starved the cells (taking away nutrients, indicated by the "-") or gave them high nutrient amount (denoted by "+").

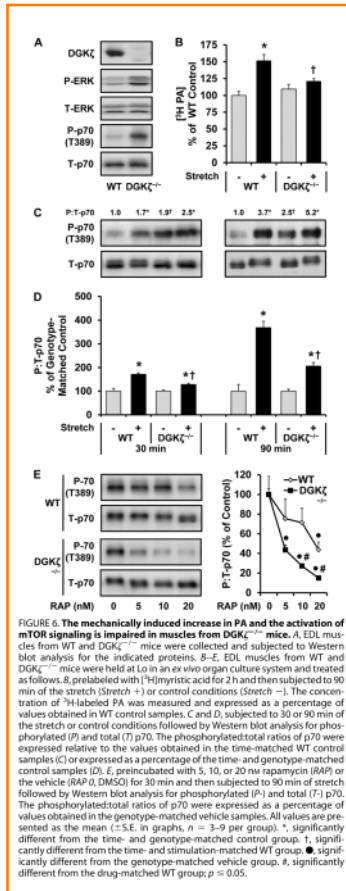
### Primary Results

- In high nutrient conditions, and with sDAGK highly expressed in cells, more phospho-p70 produced, but not with the DGK mutant (positive control) and the GFP only (negative control).

**Take Away:** In a high nutrient environment, functional sDAGK leads to more phosphorylation of p70.



### DGK $\zeta$ and Mechanotransduction in Skeletal Muscle



**FIGURE 6. The mechanically induced increase in PA and the activation of mTOR signaling is impaired in muscles from DGK $\zeta$ <sup>-/-</sup> mice.** A, EDL muscles from WT and DGK $\zeta$ <sup>-/-</sup> mice were collected and subjected to Western blot analysis for the indicated proteins. B–E, EDL muscles from WT and DGK $\zeta$ <sup>-/-</sup> mice were held at Lo in an ex vivo organ culture system and treated as follows. B, prelabeled with [<sup>3</sup>H]myristic acid for 2 h and then subjected to 90 min of the stretch (Stretch +) or control conditions (Stretch -). The concentration of <sup>3</sup>H-labeled PA was measured and expressed as a percentage of values obtained in WT control samples. C and D, subjected to 30 or 90 min of the stretch or control conditions followed by Western blot analysis for phosphorylated (P) and total (T) p70. The phosphorylated/total ratios of p70 were expressed relative to the values obtained in the time-matched WT control samples (C) or expressed as a percentage of the time- and genotype-matched control samples (D). E, preincubated with 5, 10, or 20 nM rapamycin (RAP) or the vehicle (RAP 0, DMSO) for 30 min and then subjected to 90 min of stretch followed by Western blot analysis for phosphorylated (P-) and total (T) p70. The phosphorylated/total ratios of p70 were expressed as a percentage of values obtained in the genotype-matched vehicle samples. All values are presented as the mean ( $\pm$  S.E. in graphs, n = 3–9 per group). \*, significantly different from the time- and genotype-matched control group. †, significantly different from the time- and stimulation-matched WT group. #, significantly different from the genotype-matched vehicle group. #, significantly different from the drug-matched WT group; p  $\leq$  0.05.

expected, the overexpression of WT-DGK $\zeta$ , but not KD-DGK $\zeta$ , led to an enhanced synthesis of PA as assessed by an *in vitro* DGK activity assay (Fig. 7C). Furthermore, the examination of CSA revealed that the WT-DGK $\zeta$ -transfected fibers were 33% larger than non-transfected fibers of the same muscles, whereas the CSA of fibers transfected with KD-DGK $\zeta$  or GFP was not altered (Fig. 7, A and B). Based on these results, it can be concluded that the overexpression of DGK $\zeta$  is sufficient to induce hypertrophy, and this effect requires the kinase activity of DGK $\zeta$  (i.e. the synthesis of PA).

Next, we wanted to determine if DGK $\zeta$  induces hypertrophy through an mTOR-dependent mechanism. To this end, mouse TA muscles were transfected with WT-DGK $\zeta$  or Rheb as a positive control. Immediately after transfection, the mice were subjected to daily injections of rapamycin or the solvent vehicle as a control condition. As shown in Fig. 7, D and E, a daily dose of 1.5 mg/kg rapamycin prevented the robust hypertrophic response that is induced by the overexpression of Rheb. However, the same dose of rapamycin did not significantly inhibit the hypertrophic effect of WT-DGK $\zeta$  (Fig. 7, D and E). This result was not entirely surprising because the fibers transfected with WT-DGK $\zeta$  would presumably have higher levels of PA which in turn would be expected to confer resistance to the inhibitory effects of rapamycin. Therefore, we performed an additional experiment in which the mice were injected with a higher dose of rapamycin (4.5 mg/kg/day). In this case the results demonstrated that the DGK $\zeta$ -induced hypertrophic response was significantly inhibited (Fig. 7, D and E). Thus, it appears that the DGK $\zeta$ -induced hypertrophic response is driven at least in part through an mTOR-dependent mechanism. Furthermore, just like the mechanical activation of mTOR signaling, the DGK $\zeta$ -induced hypertrophic response is partially resistant to the inhibitory effects of rapamycin, which suggests that it is mediated by an enhanced binding of PA to mTOR.

### DISCUSSION

How mechanical stimuli are converted into the molecular events that regulate skeletal muscle mass has been one of the long-standing questions in the field of muscle biology. Accordingly, several hypotheses have been proposed to explain this phenomenon, and one that has attracted increasing interest predicts that a mechanically induced increase in PA leads to an enhanced binding of PA to mTOR and in turn promotes the activation of mTOR signaling and ultimately muscle growth (4). In this study we have obtained several novel lines of evidence that support this hypothesis by identifying that (i) mechanical stimulation induces an increase in PA via a DGK $\zeta$ -dependent mechanism, (ii) loss of DGK $\zeta$  impairs mechanically induced increases in both mTOR signaling and a sign of enhanced PA-mTOR binding, and (iii) overexpression of DGK $\zeta$  promotes fiber hypertrophy through a mTOR-dependent mechanism that requires the ability of DGK $\zeta$  to synthesize PA.

In the early 2000s, a potential role for PLD and PA in the regulation of mTOR signaling was unveiled by studies that used primary alcohols (e.g. 1-butanol) to inhibit the PLD-catalyzed synthesis of PA. For example, these studies concluded that the PLD-dependent synthesis of PA is necessary for the serum-

**Figure 6:** Researchers created mice that did not have the capacity to produce sDGK (but could still produce other DGK variations/isoforms) and measured signaling proteins within the muscles of these mice compared to mice that were perfectly normal (expressing all forms of DGK). [A] The researchers are measuring if sDGK is truly "knocked out" in sDGK deficient mice and truly present in normal mice. They also measure other mTOR effectors/stimulators known as phosphorylated ERK and the already seen (in other figures) phospho-p70. [B] The researchers are measuring phosphatidic acid production in the normal mice (WT) and the sDGK deficient mice with and without stretch on the muscle. [D] Measuring phospho-p70 in normal mice and sDGK deficient mice without stretch on the muscle, as well as with stretch for 30 minutes or 90 minutes. [E] The researchers are measuring phosphorylated p70 in the normal (WT) muscle mice and the sDGK deficient muscle, but they've incubated the muscle with increasing concentrations of rapamycin (RAP), which is an mTOR inhibitor.

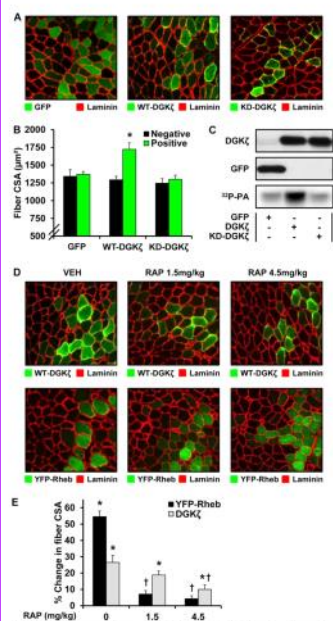
### Primary Results

- phospho-p70 is still phosphorylated when sDGK is knocked out/not present in muscle.
- phospho-ERK is increased with sDGK knockout.
- Phosphatidic acid production is blunted by the loss of sDGK
- phospho-p70 is blunted with the knockout of sDGK.
- Rapamycin decreases phosphorylation of p70 in both conditions, but more so in the sDGK deficient mice.

**Take Away:** This data implies sDGK is not necessary for the phosphorylation of p70 unless stretch is induced on the muscle (even then, it is dampened if not present), but is necessary for major production of phosphatidic acid. The loss of sDGK decreases the ability for mTOR dependent phosphorylation of p70 (likely due to lower phosphatidic acid present).

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### DGK $\zeta$ and Mechanotransduction in Skeletal Muscle



**FIGURE 7. Overexpression of DGK $\zeta$  induces hypertrophy through a kinase-dependent and rapamycin-sensitive mechanism.** A–C, mouse TA muscles were transfected with plasmid DNA encoding GFP, HA-WT-DGK $\zeta$ , or HA-KD-DGK $\zeta$ . A, the muscles were collected at 7 days post-transfection, and cross-sections from the mid-belly of the muscles were subjected to immunohistochemistry for GFP and laminin or for HA and laminin. B, CSA of the transfected fibers (Positive) and non-transfected fibers (Negative) within each of the GFP-, HA-WT-DGK $\zeta$ -, and HA-KD-DGK $\zeta$ -transfected muscles. C, muscles were collected at 3 days post-transfection, and whole lysates were subjected to a DGK activity assay (<sup>32</sup>P-PA) or Western blot analysis for the indicated proteins. D and E, mouse TA muscles were transfected with plasmid DNA encoding HA-WT-DGK $\zeta$  or YFP-Rheb. Immediately after transfection the mice were subjected to a regime of daily 1.5 or 4.5 mg/kg rapamycin (RAP) or vehicle (RAP 0, DMSO) injections. D, the muscles were collected at 7 days post-transfection, and cross-sections from the mid-belly of the muscles were subjected to immunohistochemistry for HA and laminin or for YFP and laminin. E, the CSA of the transfected fibers and non-transfected fibers within each of the HA-WT-DGK $\zeta$ - and YFP-Rheb-transfected muscles was determined, and then the percent difference between the averaged CSA of the transfected fibers and non-transfected fibers of each muscle was calculated. All values are presented as the mean  $\pm$  S.E. (n = 3–8 muscles per group, 35–120 fibers per muscle). \*, significantly different from the drug- and plasmid-matched non-transfected fibers. †, significantly different from the plasmid-matched vehicle group. p < 0.05.

phenylephrine-induced activation of mTOR signaling (18, 39). Based on these studies, it was subsequently hypothesized that the mechanical activation of mTOR signaling might also be mediated by the PLD-dependent synthesis of PA. Indeed, this appeared to be confirmed when it was demonstrated that 1-butanol can prevent mechanically induced increases in both PA and mTOR signaling (10). However, the involvement of PLD in these events became uncertain when it was shown that a newly developed PLD-specific inhibitor (FIP1) did not affect many of the biological processes that are inhibited by 1-butanol (19, 20). Moreover, a recent study has also shown that several ethanol (a two-carbon primary alcohol)-sensitive events were not observed when more reliable techniques, such as a genetic deletion of PLD, were employed (21). Consistent with the concerns raised by these studies, we found that, although FIP1 could block agonist-induced changes in PLD activity, it did not prevent the mechanically induced increase in PA or mTOR signaling. Therefore, we have concluded that the previously reported effects of 1-butanol on mechanically induced changes in PA and mTOR signaling were most likely due to off-target/PLD-independent events.

Despite the wide use of primary alcohols, there have been many studies that did not rely solely on primary alcohols to modulate PLD activity, and these studies still demonstrate that PLD can play an important role for the regulation of mTOR signaling. For example, using shRNA-mediated knockdown of PLD1, it has been shown that an increase in PLD1 activity is required for the activation of mTOR signaling that occurs during myoblast differentiation and in response to amino acid stimulation (40, 41). A more recent study has also found that modulations of PLD1 expression are sufficient to induce changes in both mTOR signaling and the size of L6 myotubes (42). Interestingly, this study also determined that, like DGK $\zeta$ , overexpression of PLD1 is sufficient to induce hypertrophy *in vivo*. Although the link between the regulation of PLD and muscle mass under physiologically relevant conditions is still not known, these results provide fundamental support for the conclusion that an elevation in PA can induce the activation of mTOR signaling and ultimately hypertrophy of skeletal muscle.

Our finding that DGK $\zeta$  contributes to the mechanical activation of PA-mTOR signaling is novel; however, the mechanism through which mechanical stimuli use DGK $\zeta$  to promote an increase in PA and mTOR signaling remains unknown. Nevertheless, recent progress in mTOR biology may have revealed some important clues. Specifically, it has been demonstrated that mTOR can colocalize with late endosomal/lysosomal structures (LELs) where its direct activators, such as Rheb, reside (24, 41, 43, 44). Furthermore, it has been shown that forced targeting of mTOR to the LEL is sufficient to activate mTOR signaling, indicating that the LEL is a key integration site for the regulation of mTOR signaling (43, 45). More recently, it was also shown that the LEL is highly enriched with PA and that mechanical stimulation leads to an increase in the colocalization of mTOR with the LEL (24, 46). This latter observation is of particular interest because it suggests that mechanical stimulation could induce an enhanced binding of PA to mTOR at least in part by increasing the association of mTOR with the PA-enriched LEL.

**Figure 7:** The researchers added a gene to muscle cells that contains the genetic material for a green fluorescent protein (GFP) attached to a gene for the specific diacylglycerol kinase of interest (sDAGK from before), known as WT-DGK. However, they also added a mutated diacylglycerol kinase that does not function, known as KD-DGK. Now, the cells will create green fluorescent protein attached to diacylglycerol kinases of one sort or another. Laminin is the definition of the cell - in red. [B] The researchers are measuring muscle cell size (Fiber CSA) in all three conditions; GFP: Control, no change in DGK; WT-DGK, increased DGK; KD-DGK, mutated, non functional DGK. [E] The researchers then wanted to know if potential increases in muscle cell/fiber size are mTOR dependent, so they added an mTOR stimulator (YFP-Rheb) or DGK and then used increasing concentrations of the mTOR inhibitor (Rapamycin, RAP).

#### Primary Results

- Expression of functional DGK has greater increases in muscle cell size.
- Rapamycin completely inhibits Rheb induced increase in muscle cell size.
- Rapamycin reduces the effect seen on increased muscle cell size in DGK expressing muscle.

**Take Away:** DGK is associated with increase muscle cell size, in at least a partially mTOR dependent manner.

Based on the information mentioned above, it is tempting to suggest that DGK $\zeta$  could regulate PA-mTOR signaling by directly controlling the level of PA at the LEL. In support of this possibility, it has been reported that DGK $\zeta$  is able to physically interact with  $\beta$ -arrestins and sorting nexin 27 (SNX27), both of which are found on early endosomal structures (47, 48). Because early endosomes can transform into LEL, the recruitment of DGK $\zeta$  into early endosomes by  $\beta$ -arrestins and/or SNX27 might ultimately contribute to an enrichment of PA at the newly formed LEL. Interestingly, it has been shown that mechanical stretching of bladder tissues results in a very rapid and robust increase in the endocytosis of the apical membrane, which is then delivered to LEL for degradation (49). Because endocytosis transports  $\beta$ -arrestins and its associated proteins (e.g. DGK $\zeta$ ) to early endosomes, it is possible that mechanical stimulation induces an enhanced targeting of DGK $\zeta$  to early endosomes, and ultimately the LEL, by promoting endocytosis (48). Consistent with this possibility, we have recently shown that mechanical stimulation induces an increase in the number of LEL (45). Furthermore, in the current study we found that mechanical stimulation promotes an increase in membranous DGK activity, which might reflect an enhanced localization of DGK $\zeta$  with the early endosomes/LEL. If correct, such an event would be expected to promote an increase in PA at the LEL and, in-turn, an enhanced binding of PA to mTOR and ultimately the activation of mTOR signaling. Clearly, this hypothesis is quite speculative, and the exact mechanism(s) via which DGK $\zeta$  contributes to the mechanical activation of PA-mTOR signaling remains to be defined. Nonetheless, examining the potential role of the LEL in this process appears to be an area that is worthy of further investigation.

Another novel finding in this study is that overexpression of DGK $\zeta$  induces fiber hypertrophy via a mechanism that requires its kinase activity. The requirement of kinase activity is noteworthy because it suggests that the DGK $\zeta$ -induced hypertrophy was mediated by an increase in the synthesis of PA. As shown in previous studies, an enhanced synthesis of PA can trigger mTOR-dependent (rapamycin-sensitive) cellular responses, but these responses tend to be resistant to the inhibitory effects of rapamycin (10, 50). For instance, PLD activity/PA is frequently elevated in human breast cancer cells, and the growth rate of these cells becomes partially resistant to rapamycin in a manner that is correlated with the higher levels of PLD activity (50). Therefore, our observation that overexpression of DGK $\zeta$  induces hypertrophy through an mTOR-dependent (rapamycin-sensitive), but partially rapamycin-resistant mechanism, further suggests that the hypertrophic effect of DGK $\zeta$  is exerted through an increase in PA that in turn would lead to an enhanced binding of PA to mTOR and the subsequent activation of mTOR-dependent growth regulatory events.

Previous studies have demonstrated that signaling through mTOR is necessary for a mechanically induced hypertrophic response and that the activation of mTOR signaling is sufficient to induce hypertrophy (3, 5, 6). Thus, the results of this study strongly suggest that DGK $\zeta$  could also play a role in the molecular process through which mechanical stimuli regulate skeletal muscle mass. Consistent with this possibility, a recent clinical

study found that subjects who displayed the greatest degree of myofiber hypertrophy in response to heavy resistance exercise also had the highest pre-training levels of skeletal muscle DGK $\zeta$  transcript expression (51). Additional evidence implicating DGK $\zeta$  in mechanically induced hypertrophy is that DGK $\zeta$  can regulate several stages of skeletal myogenesis including myoblast differentiation and fusion, a process that has been widely implicated in the regulation of mechanically induced hypertrophy (52–54). Because the process of myogenesis is also largely controlled by mTOR signaling, it is possible that DGK $\zeta$  could regulate mechanically induced hypertrophy via the control of mTOR-dependent myogenesis (55). Combined, several pieces of evidence suggest that DGK $\zeta$  could be a key component of the mechanism(s) through which mechanical stimuli induce hypertrophy, and addressing this possibility will be an essential topic for future studies.

In summary, the results from this study indicate that DGK $\zeta$  contributes to the mechanical activation of PA-mTOR signaling and induces hypertrophy via an mTOR-dependent mechanism that requires the ability of DGK $\zeta$  to synthesize PA. In the future it will be important to determine how DGK $\zeta$  contributes to the mechanical activation of PA-mTOR signaling and to define the role of DGK $\zeta$  in mechanically induced hypertrophy *in vivo*. The resulting knowledge will further advance our understanding of the mechanism(s) through which mechanical stimuli regulate skeletal muscle mass and may ultimately lead to the development of therapies that can prevent the loss of muscle mass during conditions such as aging, diseases, and inactivity.

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**The Role of Diacylglycerol Kinase  $\zeta$  and Phosphatidic Acid in the Mechanical Activation of Mammalian Target of Rapamycin (mTOR) Signaling and Skeletal Muscle Hypertrophy**

Jae-Sung You, Hannah C. Lincoln, Chan-Ran Kim, John W. Frey, Craig A. Goodman, Xiao-Ping Zhong and Troy A. Hornberger

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